The Journal of Neuroscience
July 1986, 6(7): 1970-1975

Catecholamine, Serotonin, and Substance P-like Peptide Containing Intrinsic Neurons in the Mudpuppy Parasympathetic Cardiac Ganglion

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The mudpuppy cardiac ganglion contains 2 neuron types: large parasympathetic postganglionic projection neurons and smaller intrinsic neurons originally described by McMahan and Purves (1976) as intensely fluorescent (SIF) cells. The function of these SIF cells, present in the mudpuppy cardiac ganglion, is unknown. Further, direct application of catecholamines, which are thought to be contained in SIF cells, to the parasympathetic postganglionic cells has no effect (Hartzell et al., 1977). As SIF cells in other ganglion preparations recently have been shown to contain putative transmitter substances in addition to catecholamines, immunocytochemical experiments were conducted to test for the presence of additional transmitter substances in the SIF cells within the cardiac ganglion. Whole-mount septal preparations were dissected from *Necturus maculosus* and processed for indirect immunocytochemistry. The results indicated that many of these intrinsic neurons contained 5-HT or a substance P-like peptide, or both. Many small intrinsic neurons which contain either substance P or 5-HT were also positive for aqueous-aldehyde-induced fluorescence, indicating the presence of a catecholamine. Finally, some of these cells appeared to contain all 3: a catecholamine, 5-HT, and a substance P-like peptide.

The presence of small, intensely fluorescent (SIF) cells within autonomic ganglia of many different species has been well documented (Taxi et al., 1983; Williams and Jew, 1983). However, the function of these cells remains a matter of speculation (Williams and Jew, 1983). Because of the complex organization of most vertebrate ganglia and the small size of the SIF cells, electrophysiological recordings from SIF cells have been obtained in only a few preparations (Dunn and Marshall, 1985; Roper, 1976).

We have initiated studies to characterize the histochemical and electrophysiological properties of SIF cells within the cardiac ganglion of *Necturus*. In this ganglion preparation, there are 2 neuron types, as described by McMahan and Purves (1976): large parasympathetic postganglionic projection cells and the smaller neurons they designated as SIF cells. These 2 cell types can be visually identified in living preparations. Further, the SIF cells in this preparation are of sufficient size for obtaining intracellular recordings (Roper, 1976).

Separate populations of SIF cells have been identified in other ganglia; in each the SIF cells are thought to contain a different catecholamine or indolamine (Williams and Jew, 1983). Further, some SIF cells may contain both a catecholamine and a neuropeptide (Helèn et al., 1984; Legay et al., 1984). Therefore, as part of our initial characterization of the SIF cells within the mudpuppy cardiac ganglion, the present histochemical experiments were undertaken to investigate the possible presence of other putative transmitter substances contained in these cells.

Materials and Methods

Whole septal preparations were taken from *Necturus maculosus* (mudpuppy). This tissue sheet, which contains the cardiac parasympathetic ganglion, was removed and pinned onto Sylgard-coated petri dishes containing a HEPES-buffered physiological solution (mM: NaCl, 120; KCl, 2.5; $CaCl_2$, 1.8; HEPES, 1.0; pH 7.3) as outlined previously (Hartzell et al., 1977; McMahan and Purves, 1976; Roper, 1976). Excessive connective tissue was carefully teased away from each preparation prior to fixation. The septum was then prepared for glyoxylic acid-induced fluorescence, using the procedures outlined by Furness and Costa (1975), or for immunohistochemistry, following the methods of Costa et al. (1980).

The glyoxylic acid-induced fluorescence technique provides a reliable means of visualizing the SIF cells and other catecholamine-containing nerve fibers (Furness and Costa, 1975). For this, the preparations were immersed in 2% wt/vol glyoxylic acid in 0.1 M phosphate buffer (pH 7.0 or 3.5) at 4°C for 30 min. The preparations were then mounted on glass slides, partially air-dried, and incubated at 100°C for 4 min.

For immunocytochemistry, the isolated septal preparations were pinned on a paraffin block and immersed in fixative according to the following protocols: (1) To examine for neuropeptides or other putative neurotransmitters, the preparations were incubated in cold (4°C) Zamboni's fixative (0.1 M phosphate-buffered picric acid/formaldehyde mixture; pH 7.3) for approximately 18 hr (Costa et al., 1980) prior to exposure to primary antisera. (2) To determine coexistence of catecholamine and other transmitter-like substances, the preparations were fixed for approximately 30 min in 4% paraformaldehyde and 0.25% glutaraldehyde (Faglu fix) in 0.1 M PBS, pH 7.4 (Furness et al., 1978). These Faglu-fixed preparations were used for combined aqueous aldehyde-induced fluorescence and immunohistochemical staining (Helèn et al., 1984).

Fixed whole-mount preparations were processed for indirect immunohistochemistry (Coons, 1958). The preparations were incubated with primary antisera overnight at room temperature, washed with PBS, and then incubated 1 hr with a fluorescent-tagged secondary antisera. Triton X-100 (0.3%) and 1% normal goat serum were included in the incubating solutions. The preparations were then washed in buffer, transferred to slides, and mounted in carbonate-buffered 50% glycerol (pH 8.6). Substance P-immunoreactive neurons and fiber processes were visualized using a rat monoclonal antisera (Sera Labs) at dilutions of 1:100 or 1:200, followed by exposure to either a fluorescein-isothiocyanate (FITC)-conjugated goat anti-rat IgG (Cappel; 1:40 dilution) or a tetramethylrhodamine conjugated goat anti-rat IgG (Cappel; 1:40 dilution). Neurons and processes immunoreactive for 5-HT were visualized using an antisera raised in rabbit against 5-HT (Immunonuclear Corp.) at dilutions of 1:100 to 1:500, followed by incubation in either a FITC-conjugated goat anti-rabbit antiserum (1:40) or a rhodamine-conjugated goat anti-rabbit antiserum (1:40).

Control preparations were incubated with either secondary antisera

Received Aug. 12, 1985; revised Dec. 13, 1985; accepted Jan. 13, 1986.

We thank Drs. Marjorie Ariano and Richard Kriebel for technical guidance and helpful discussions during the course of this study. We also wish to thank Dr. Ariano for the use of her Zeiss photomicroscope in the preparation of all the photomicrographs presented in this report. Partial support for this project was obtained from USPHS Grant NS-19880 to R.L.P.

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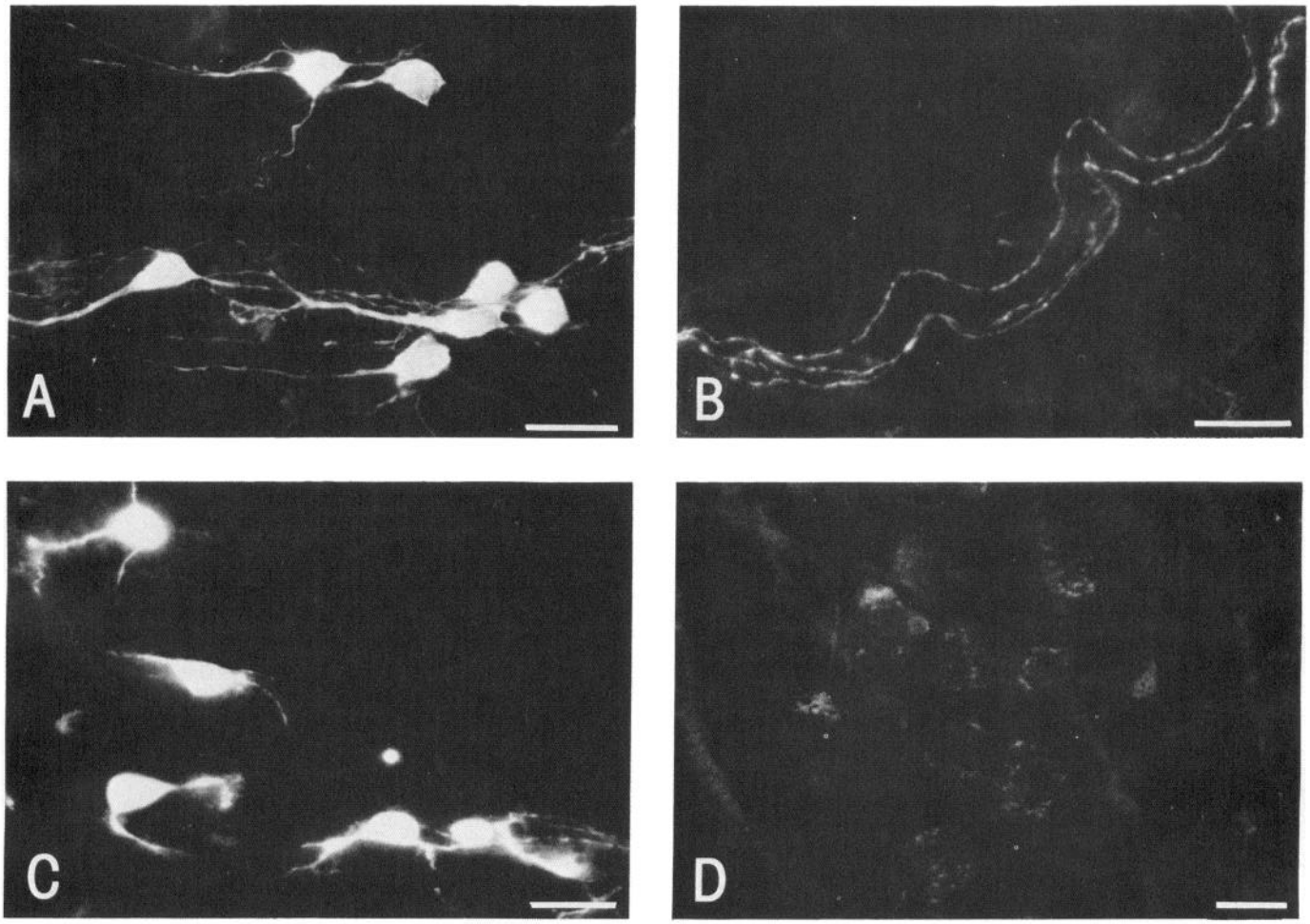

Figure 1. Comparison of glyoxylic acid-induced fluorescence at pH 7.2 (*A, B*) or pH 3.5 (*C, D*). *A,* A group of SIF cells. *B,* An example of the numerous bundles of long fluorescent fibers that course across the septum and form complexes over strands of cardiac muscle and clusters of postganglionic cells. *C,* Numerous intrinsic neurons are fluorescent after glyoxylic acid fixation at pH 3.5. However, as shown in *D,* which represents an area containing strands of cardiac muscle and a group of ganglion cells, there are no long fibers exhibiting fluorescence after fixation with glyoxylic acid buffered to pH 3.5. Calibration bars: *A, B,* and *D,* 40 μm; *C,* 30 μm.

alone or with primary antisera that had been preabsorbed with excess substance P triacetate (Sigma) or a 5-HT BSA conjugate (Immunonuclear Corp.). These preparations exhibited no fluorescent-labeled cells or fibers.

Each whole-mount preparation was examined with a Zeiss fluorescence photomicroscope equipped with an HBO 100 W UV light source. A 360 nm primary filter/405 nm secondary filter combination was used to visualize aldehyde-induced or glyoxylic acid-induced fluorescence; a 565 nm primary filter/590 nm secondary filter combination was used to visualize rhodamine-labeled cells; and a 485 nm primary filter/520 nm secondary filter combination was used to visualize FITC-labeled cells. Photomicrography was performed with Ilford HP-5 black and white film, push-processed one stop, using Kodak D-19 to increase the contrast of the negative.

Results

SIF neurons in the cardiac ganglion

McMahan and Purves (1976) have previously demonstrated the presence of SIF cells in the mudpuppy cardiac ganglion using the Falck-Hillarp procedure for catecholamine localization. We have obtained similar results with the glyoxylic acid method of Furness and Costa (1975). Numerous SIF cells are present within clusters of the larger principal postganglionic neurons that lie along the vagal trunk (Fig. 1*A*). These glyoxylic acid-fluorescent cells vary in shape and number of processes. Generally, the SIF cell processes extend 100–200 μm into the region of adjacent ganglion cells, but in a few instances longer processes extending several hundred microns were observed. Long catecholamine-containing axons from postganglionic sympathetic neurons also are fluorescent in the glyoxylic acid-fixed (pH 7.0) preparations (Fig. 1*B*). These course across the septal tissue sheet to innervate cardiac muscle, and often are intermingled with processes of the SIF cells within the clusters of ganglion cells.

Recently, separate populations of small, 5-HT- and catecholamine-containing cells have been identified in other ganglion preparations (Verhofstad et al., 1981). We also took advantage of the glyoxylic acid technique to test whether both catecholaminergic and serotinergic intrinsic neurons might be present as well in the mudpuppy cardiac ganglion. In our initial experiments, the glyoxylic acid solution was buffered to pH 7.0. At this pH, both catecholamines and indolamines are fluorescent in glyoxylic acid-fixed preparations. However, at pH 3.5, 5-HT forms a fluorophore but the catecholamines do not (Furness and Costa, 1975). Therefore, preparations were fixed in the glyoxylic acid solution buffered to pH 3.5. There were many fluorescent intrinsic neurons in these preparations (Fig. 1*C*), and the sizes, shapes, and extents of their processes were similar to those of the cells fluorescent at pH 7.0. In contrast, at pH 3.5,

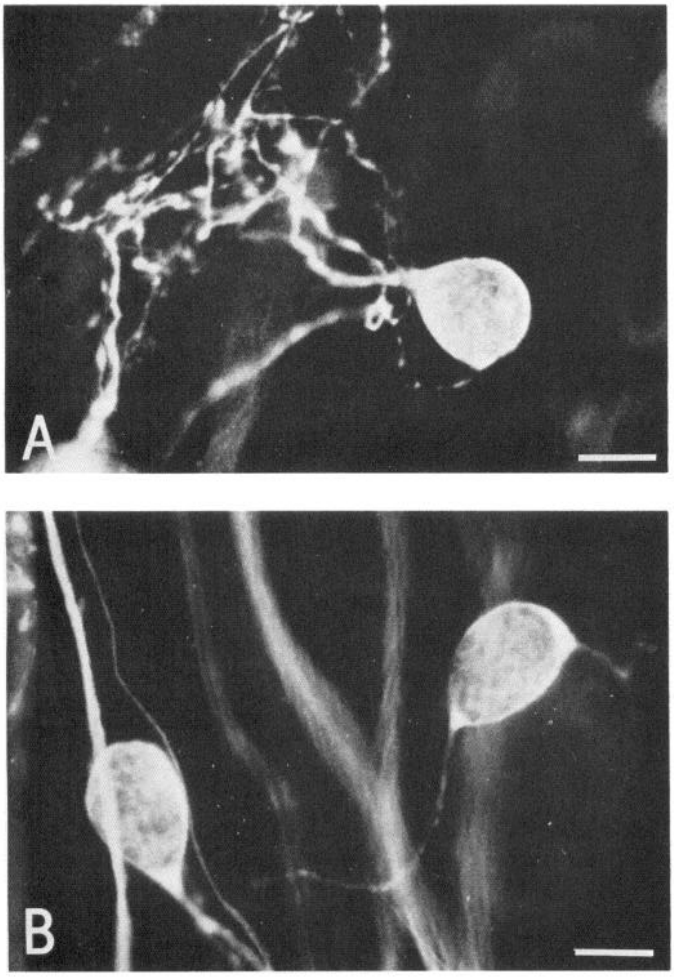

Figure 2. Examples from 2 different Zamboni-fixed preparations of intrinsic neurons immunoreactive for 5-HT or a substance P-like peptide. *A*, A cell and its processes immunoreactive for 5-HT (FITC-labeled secondary antisera). *B*, Two cells and adjacent long fiber processes immunoreactive for substance P (FITC-labeled secondary antisera). Calibration bars, 30 μm.

none of the catecholamine-containing long postganglionic sympathetic axons was fluorescent (Fig. 1*D*). These results suggested that many intrinsic neurons in the mudpuppy cardiac ganglion may contain 5-HT.

Serotonin-immunoreactive intrinsic neurons in the cardiac ganglion

Immunocytochemical experiments were conducted to verify the presence of 5-HT-containing intrinsic neurons. Our results indicated that numerous 5-HT-reactive cells were present in the cardiac ganglion (Fig. 2*A*). Both the soma and processes of these cells were well labeled and these 5-HT-immunoreactive cells were similar in size, shape, and number of processes to those exhibiting fluorescence following glyoxylic acid fixation. The 5-HT immunoreactivity was absent when the 5-HT antisera (1:200) was preincubated with 200 μg/ml of a 5-HT–BSA conjugate (Immunonuclear Corp.).

Substance P-immunoreactive intrinsic neurons in the cardiac ganglion

Many small intrinsic neurons with substance P immunoreactivity were also identified among nonreactive postganglionic parasympathetic cells (Fig. 2*B*). Processes from some of these substance P-reactive cells appeared to form complex pericellular appositions around adjacent parasympathetic ganglion cells. These substance P-reactive neurons were similar in size and shape to the glyoxylic acid fluorescent cells and to cells exhibiting 5-HT immunoreactivity. In addition, there were many other substance P-immunoreactive fiber processes within the cardiac ganglion which were not derived from intraganglionic neurons. Therefore, it was often difficult to determine the length and distribution of the processes from the substance P-immunoreactive intrinsic neurons. The source of these extrinsic substance P-reactive fibers, i.e., parasympathetic preganglionic (Bowers et al., 1984) or sensory afferent (Hökfelt et al., 1977; Matthews and Cuello, 1982) fibers is not known at present. The substance P immunoreactivity of cells and fibers within the cardiac ganglion was absent when antisera (1:200) preabsorbed with substance P (300 μg/ml) were used.

The substance P-like peptide and 5-HT are both present in some intrinsic neurons

Six Zamboni-fixed septal preparations were "double-labeled" to test whether both 5-HT and substance P immunoreactivity was present in individual cells. The secondary antisera were conjugated to different fluorochromes—rhodamine for 5-HT immunoreactivity and FITC for substance P immunoreactivity (Fig. 3). Quantitative cell counts were made on 4 of these preparations (total of 659 cells). The results indicated that approximately 5% of the total number of reactive cells was immunoreactive for only substance P, and approximately 31% of the reactive cells exhibited only 5-HT immunoreactivity. However, 64% of the reactive cells exhibited both 5-HT immunoreactivity and substance P immunoreactivity.

Co-localization of substance P-like peptide, 5-HT, and catecholamine in individual intrinsic neurons

Combined procedures for catecholamine and peptide localization indicated that catecholamine and substance P-like peptide may be contained in the same intrinsic neurons. In Faglu-fixed preparations the soma of many individual intrinsic neurons exhibited a bright blue–white fluorescence, whereas none of the principal cells exhibited any catecholamine fluorescence (Fig. 4*A*). In most instances, the processes of individual intrinsic neurons were too faintly fluorescent to be clearly distinguishable. Five preparations were tested for the presence of both catecholamine and substance P-like peptide in individual cells. A rhodamine-labeled secondary antiserum was used in order to distinguish aqueous aldehyde-induced fluorescence of catecholamine-containing cells from indirect fluorescence for peptide (Fig. 4, *B, C*). Cell counts from 3 of these preparations (a total of 175 cells) indicated that 6% of the visually identifiable intrinsic neurons exhibited only substance P immunoreactivity, 54% exhibited only catecholamine fluorescence, and 40% exhibited both positive catecholamine fluorescence and substance P immunoreactivity.

Evidence has also been obtained for the coexistence of 5-HT and catecholamine in some intrinsic neurons. Other septal preparations, which were fixed in the paraformaldehyde–glutaraldehyde solution, were examined for 5-HT-immunoreactive neurons (Fig. 5). In 3 preparations (a total of 150 cells) analyzed for possible colocalization of catecholamine and 5-HT, 4% of the visually identifiable intrinsic neurons exhibited only catecholamine fluorescence, approximately 25% were immunoreactive only to 5-HT without any discernible catecholamine fluorescence, and 71% exhibited a positive reaction for catecholamine and immunoreactivity for 5-HT.

A number of experiments were done to test whether individual intrinsic neurons were reactive for all three—a catecholamine, 5-HT, and substance P. For this analysis, preparations were also fixed in the paraformaldehyde–glutaraldehyde solution to access catecholamine presence by aqueous-aldehyde-

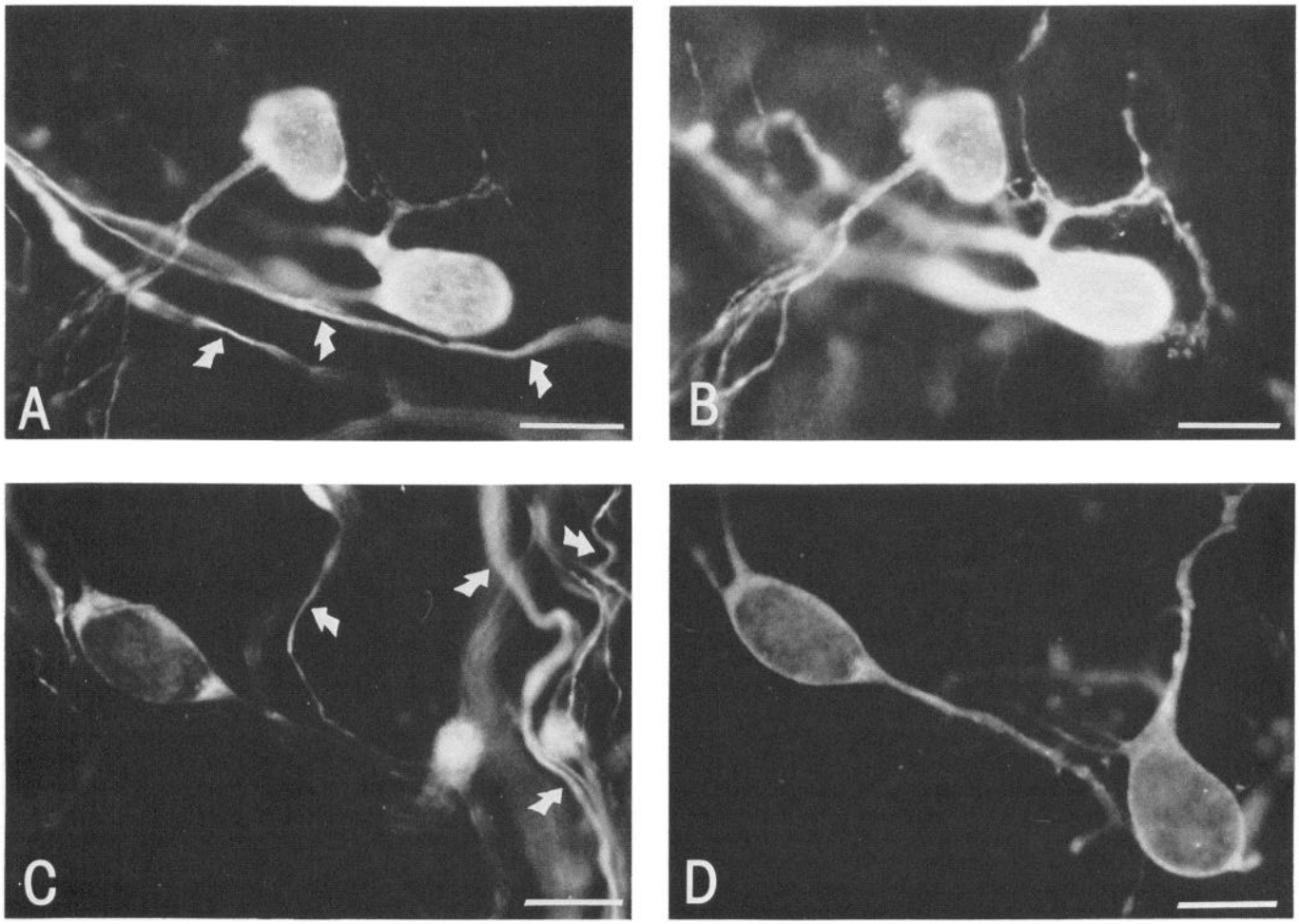

Figure 3. Evidence from 2 different Zamboni-fixed septal preparations for the presence of both 5-HT and a substance P-like peptide within individual intrinsic neurons. *A,* Two cells and long fiber processes immunoreactive for substance P (FITC-labeled secondary antisera). *B,* These same 2 cells also exhibit immunoreactivity for 5-HT (rhodamine-labeled secondary antisera). Note that the long fiber processes present in *A* (indicated by the *arrows*) are not immunoreactive for 5-HT. *C,* A cell and adjacent long fiber processes (indicated by *arrows*) immunoreactive for substance P (FITC-labeled secondary antisera). *D,* The cell shown in *C,* as well as an adjacent cell, exhibits immunoreactivity for 5-HT (rhodamine-labeled secondary antisera). Calibration bars, 25 μm.

induced fluorescence. A rhodamine-labeled secondary antibody was used for the determination of the 5-HT immunoreactivity and a FITC-conjugated secondary antibody was used for substance P immunoreactivity. The results demonstrated that many of these small cells exhibited fluorescence for catecholamine and immunoreactivity for both a substance P-like peptide and 5-HT (Fig. 6). The number of cells in this category varied over a wide (10–50%) range in different preparations. Further, in these preparations, colocalizations as described above were also evident.

Discussion

McMahan and Purves reported in 1976 that there were 2 neuron types in the mudpuppy parasympathetic cardiac ganglion: large parasympathetic postganglionic neurons and smaller, catecholamine-containing fluorescent cells. However, the function of the SIF cells is still unknown. Dopamine was suggested as being the major catecholamine in the SIF cells of the mudpuppy cardiac ganglion (Hartzell et al., 1977). However, the application of dopamine, epinephrine, or norepinephrine was found to be without effect on the principal postganglionic neurons (Hartzell et al., 1977). The present results demonstrate that many of the small intrinsic neurons in the cardiac ganglion also exhibit immunoreactivity for 5-HT or a substance P-like peptide. The inability to demonstrate any direct effect of catecholamines on the postganglionic neurons may mean that the substance P-like peptide or 5-HT, rather than the catecholamines, is responsible for either neurotransmitter or neuromodulatory actions. Alternatively, all of these substances may be involved in integration within the ganglion, but at different sites. Each could have either pre- or postsynaptic sites of action, thereby modifying transmitter release from preganglionic fibers or directly altering the electrical properties of postganglionic cells. The resolution to this problem requires additional experiments investigating the roles of these putative transmitter substances in ganglionic transmission as well as in the properties of the postganglionic neurons.

The coexistence of a "classical" transmitter such as dopamine or 5-HT with a neuropeptide is now well documented for many different CNS and peripheral nervous system neurons (Legay et al., 1984; Lundberg and Hökfelt, 1983). We have observed in the mudpuppy cardiac ganglion that many individual intrinsic neurons may contain catecholamine, 5-HT, a substance P-like peptide, or some combination of each. One unusual aspect of the present observations is the possibility that a catecholamine and an indolamine may be colocalized within the same cell. However, we recognize that the interpretation of results from histochemical procedures such as the aqueous-aldehyde-induced fluorescence technique needs to be guarded. For example,

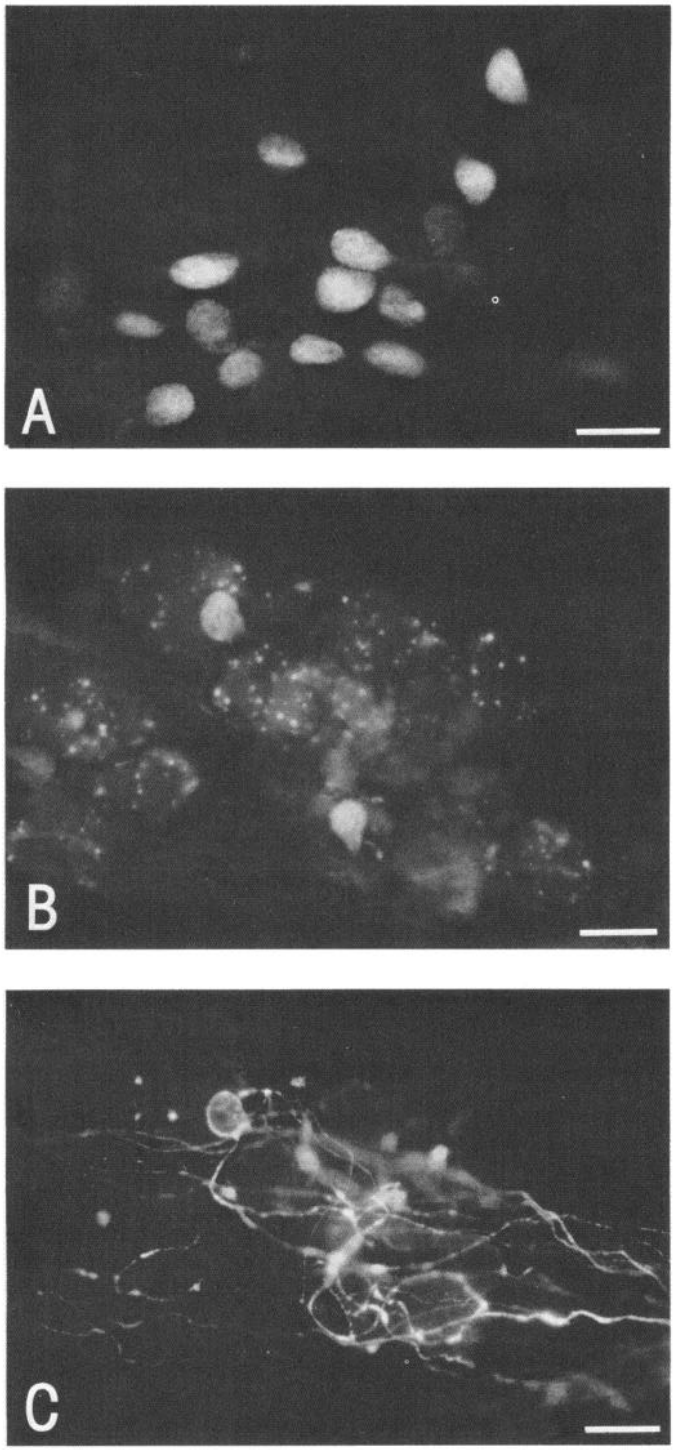

Figure 4. Evidence from Faglu-fixed preparations for the colocalization of a substance P-like peptide and a catecholamine within individual cells. *A*, A cluster of cells exhibiting fluorescence intermingled among nonfluorescent postganglionic neurons. *B*, An example of 2 intrinsic neurons fluorescent for catecholamine among a group of principal cells. *C*, Photograph of the same area as in *B*, illustrating that one of these 2 neurons is also immunoreactive for substance P (rhodamine-labeled secondary antisera). Note also the presence of numerous substance P fiber processes complexing around this cluster of principal cells. Calibration bars: *A*, 40 μm; *B* and *C*, 50 μm.

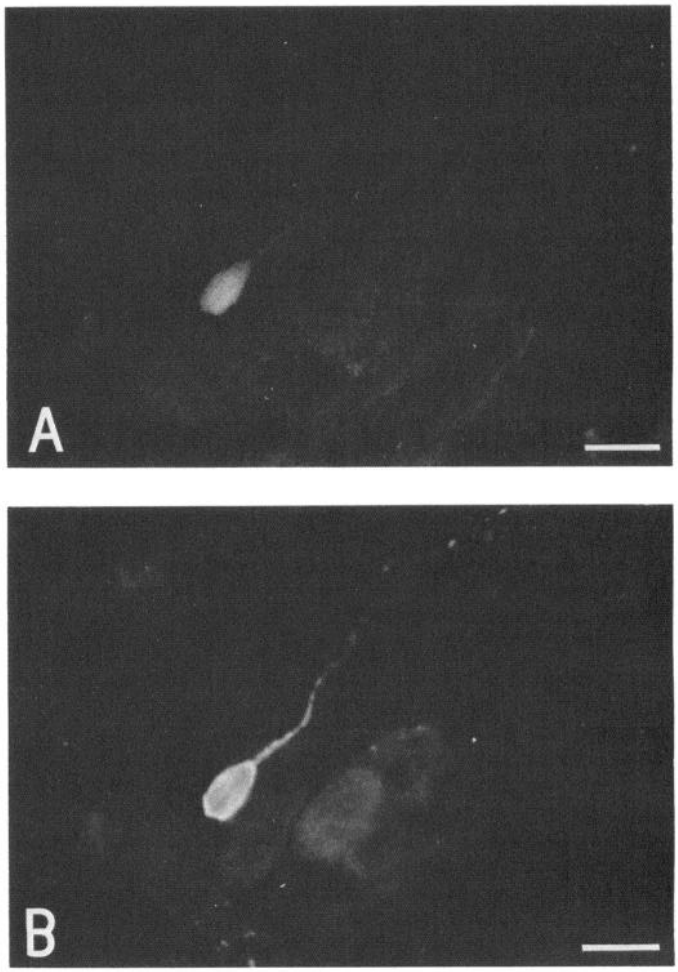

Figure 5. Evidence from a Faglu-fixed preparation indicating that an individual intrinsic neuron can exhibit both catecholamine fluorescence (*A*) and 5-HT immunoreactivity (*B*). For the 5-HT immunoreactivity, a rhodamine-labeled secondary antiserum was used. Calibration bars, 40 μm.

we have assumed, on the basis of previously published observations, that the bluish-white fluorescence in Faglu-fixed tissue was indicative of the presence of a catecholamine rather than of 5-HT, which has a yellowish fluorescence. Further, other investigators have reported that the 5-HT fluorescence is often more difficult to obtain than catecholamine fluorescence in Faglu-fixed tissue (Witkovsky et al., 1984). The validity of our assumptions concerning the septal tissue is crucial to the conclusion that both a catecholamine and an indolamine may be present within an individual cell.

Given these possible limitations in technique, our results demonstrate that a number of different putative transmitter substances are present within intrinsic neurons of the mudpuppy cardiac ganglion. These observations are of particular interest for 2 reasons: First of all, the anatomical features of the above preparation make it ideal for future studies of the role of peptides and other intrinsic transmitters in integration within a parasympathetic ganglion. Second, the septal tissue sheet containing the cardiac ganglion can be isolated and maintained for prolonged periods under controlled environmental conditions as an explant in organ culture (Neel and Parsons, unpublished observations; Roper, 1976). Therefore, this preparation should also be valuable in other studies designed to establish those factors that regulate the production of different potential transmitter substances within a defined cell type.

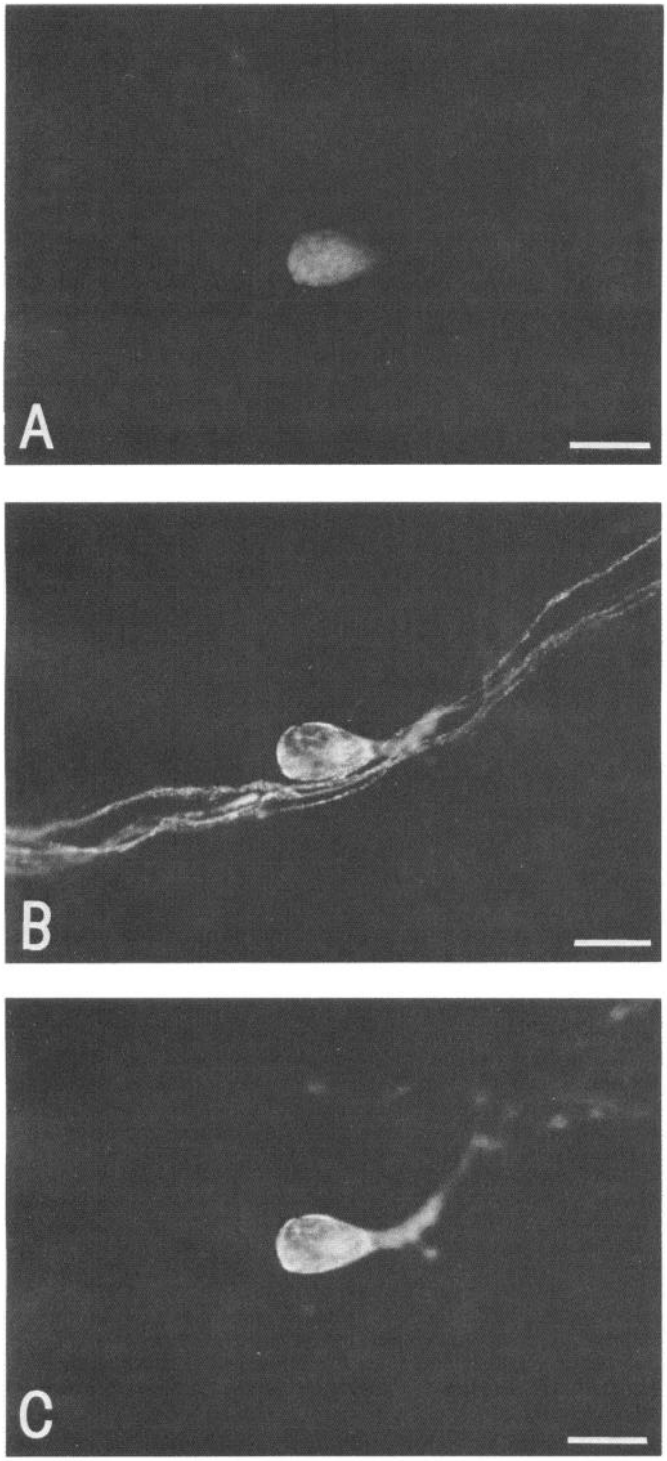

Figure 6. Evidence obtained from a Faglu-fixed preparation for the presence of a catecholamine, substance P-like peptide, and 5-HT in an individual intrinsic neuron. *A,* A cell exhibits catecholamine fluorescence. *B,* This same cell exhibits immunoreactivity for substance P (FITC-labeled secondary antisera). *C,* This cell (but not the long fiber processes) also exhibits immunoreactivity for 5-HT (rhodamine-labeled secondary antisera). Note that the bright substance P-like immunoreactive long fiber processes do not exhibit fluorescence in *A* or *C,* indicating the effectiveness of the filter combinations. Calibration bars, 30 μm.

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